Journal of Medical Virology 53:266–272 (1997)

Sequential Analyses of the Mutations in the Core Upstream and Precore Regions of Hepatitis B Virus Genome in Anti-HBe Positive-Carriers Developing Acute Exacerbation

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The nucleotide sequences of the core upstream and precore regions (371 nucleotide length, nt. 1604-1974) of hepatitis B virus (HBV) were analysed sequentially in three subjects who were positive serorogically for anti-HBe and had acute clinical exacerbation after immunosuppressive treatment. These patients were asymptomatic HBV carriers before therapy. The results revealed that the mutant with an 8-bp deletion (nt. 1768–1775) located in the basic core promoter region was dominant in the asymptomatic HBV carrier phase in two of three subjects. After exacerbation, however, such mutant clones possessing 8-bp deletion disappeared or decreased in number and were replaced by the clones possessing a precore stop codon mutation G to A (nt. 1896) or by the clones possessing additional contiguous point mutations A to T (nt. 1762) and G to A (nt. 1764) and a new point mutation C to T (nt. 1653). Possible relationships between acute exacerbation of liver function accompanied by mutation and the transition of the dominant clones were discussed. ***J. Med. Virol. 53: 266–272, 1997.***

KEY WORDS: hepatitis B virus; core promoter; mutation; acute exacerbation

INTRODUCTION

Recent studies concerning mutant hepatitis B virus (HBV) genomes have revealed that the emergence of precore defective mutant HBV, which is characterized by a point mutation from G to A at nucleotide (nt.) 1896, not only corresponds to the seroconversion of hepatitis B e antigen (HBeAg) to anti-HBe but also to severe hepatic damage, including fulminant hepatitis and acute exacerbation of chronic hepatitis B [Carman et al., 1989; Liang et al., 1991; Okamoto et al., 1990; Omata et al., 1991]. In the natural course of HBV infection, the replication of HBV is generally thought to cease after the individuals infected with HBV convert to anti-HBe. On the other hand, such a naturally occurring precore defective mutant, with a G to A mutation at nt. 1896, has been reported to enhance the stability of the stem-loop structure in the encapsidation signal and to ensure viral replication [Lok et al., 1994; Tong et al., 1992]. In spite of low replicative competence clinically, such an HBV mutant has the potential of restoring replicative activity under some conditions, such as during an immunosuppressive state or during the administration of immunosuppressive drugs [Lok et al., 1991].

More recently, several investigators reported the presence of various mutations in the core upstream and the X gene regions which are thought to be important for the viral life cycle [Fukuda et al., 1995; Gotoh et al., 1995; Laskus et al., 1994; Nishizono et al., 1995; Okamoto et al., 1994; Uchida et al., 1995]. The present study was undertaken to discover the possible relationships between the mutations located in the core upstream and precore regions which overlap with the X gene (hereafter referred as the target region) and viral replication. We therefore sequenced the target region of HBV genomes obtained from three anti-HBe positive carriers during the clinical phases of asymptomatic carriage, acute exacerbation following immunosuppressive therapy, and post-exacerbation.

MATERIALS AND METHODS

Patients and Samples

All cases were anti-HBe positive carriers (subtype *adr*) who were suffering from several underlying dis-

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Accepted 9 July 1997

eases apart from the HBV infection and who developed acute exacerbation after immunosuppressive therapy (Table I). Informed consent was obtained from all patients.

Case 1 was a 58-year-old Japanese man with non-Hodgkin's lymphoma, B cell, diffuse and medium type. He attended the outpatient clinic and his alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels had remained within normal range until his admission. Serologically, he was positive for HBsAg (5120× PHA titer), negative for HBeAg, but positive for anti-HBe. A serum sample for nucleotide sequencing during the asymptomatic phase was taken on admission. Chemotherapy to combat his condition, consisting of pirarubicin hydrochloride (THP, 70 mg/day), cyclophosphamide (CPA, 900 mg/day), vincristine sulfate (VCR, 1.8 mg/day) and predonisol one (PSL, 70 mg/day) started immediately after admission. Details of this chemotherapy are described in Table I. ALT and AST were elevated and reached maximum levels (ALT 1045, AST 496) two months after the first treatment. Blood sampling for sequencing during the exacerbation phase was carried out just before the transaminases reached maximum levels. Three weeks later, a liver biopsy was undertaken when the transaminase levels returned to normal (ALT 76, AST 45). Pathological findings showed acute hepatitis with diffusely distributed focal necrosis but there was no evidence of chronic changes. A serum sample for sequencing of post-exacerbation phase was taken at the same time. The patient recovered completely.

Case 2 was a 47-year-old Japanese man who was found to have a brain tumor by MRI examination. The tumor was resected. The histological findings showed glioblastoma. He was positive for HBsAg (1280× PHA titer), negative for HBeAg but positive for anti-HBe and the levels of transaminases remained within the normal range. A serum sample for sequencing during the asymptomatic carriage phase was taken before the start of chemotherapy. Chemotherapy to prevent recurrence of the tumor consisted of nimustine hydrochloride (ACNU, 250 mg/day), methotrexate (MTX, 500 mg/day), carboplatine (CBDCA, 400 mg/day), etoposide (VP-16, 300 mg/day). It was administrated for 2 days, except for PSL. PSL (125 mg/day) was given daily for 30 days. The levels of transaminases became elevated just before the end of PSL administration and reached their maximum levels (ALT 738, AST 450) one week later. Serum samples for sequencing at exacerbation and post-exacerbation phases were taken at the peak of transaminase levels and one month later when the levels declined to the normal range (ALT 21, AST 22), respectively. Throughout the clinical course, he was negative for HBeAg and positive for anti-HBe and his DNA-polymerase remained at a low level.

Case 3 was a 50-year-old Japanese woman with dermatomyositis. Serologically, she was found to be an HBsAg carrier with anti-HBe. She was given PSL (60 mg/day) at the beginning but the dose was reduced gradually over a period of 9 months. Azathioprine

TABLE I. Clinical and Laboratory Data of Three Subjects Showing Acute Exacerbation after Immunosuppressive Therapy

Case	Age (yr)	Sex	Underlying disease	Administrated drug (dose: mg/day × day × times)	HBe Ag/ anti-HBe	ASC			Exacerbation			Post-exacerbation		
						ALT	DNA-p	HBVDNA*	ALT	DNA-p	HBVDNA*	ALT	DNA-p	HBVDNA*
1	58	M	non-Hodgkin's lymphoma	THP (70 × 2) CPA (900 × 2) VCR (1.8 × 1) PSL (70 × 5) × 3†	−/+	18	<30	10^2	1045	<30	10^5	76	<30	$<10^2$
2	47	M	Recurrence of glioblastoma	ACNU (250 × 2) MTX (500 × 1) CBDCA (400 × 2) VP-16 (300 × 2) PSL (125 × 30)	−/+	49	<30	10^4	738	<30	10^8	21	<30	$<10^2$
3	50	F	Dermatomyositis	AZP (100 × 150) PSL (60 × 270)	−/+	35	<30	$<10^2$	698	9665	10^{10}	not examined due to death		

*copies/ml.
The course of chemotherapy described in bracket was repeated 3 times at approximately 1 week interval (CHOP therapy).

(AZP, 100 mg/day) was given for 5 months. Forty days after the start of therapy, a serum sample for nucleotide sequencing in ASC phase was taken, when the levels of ALT and AST still remained within normal range and the level of HBV DNA polymerase was undetectable. Blood screening carried out after 8 months from the start of therapy showed abnormal liver function (ALT 698, AST 1088, DNA polymerase 9665 cpm) and blood sampling in the exacerbated phase was performed at this time. A liver biopsy showed chronic active hepatitis 2A, with moderate infiltration with inflammatory cells in the parenchyma and portal tracts. As there was no evidence of other viral superinfection, such as delta agent or hepatitis C virus (HCV), this exacerbation of liver function was considered to be due to the reactivation of HBV. Finally, her clinical course progressed to hepatic decompensation and she died of fulminant hepatitis one month later.

Other factors which might cause liver dysfunction, such as alcohol abuse, diabetes mellitus, and autoimmune disorders were negative in all cases.

Serology and Quantitative Analysis of HBV DNA

HBsAg and the antibody to HBsAg (anti-HBs) were assayed by reverse passive hemagglutination (RPHA) and passive hemagglutination (PHA) (Ausab, Dynabot, Abbott Park, IL), respectively. HBeAg, anti-HBe and the anti-hepatitis B core antibody (anti-HBc) were assayed by RIA (Abbott Laboratories, Abbott Park, IL).

HBV DNAs were extracted from 100 μl of serum by the sodium iodide method [Ishizawa et al., 1991]. Control HBV DNA, named pHB4 (subtype adr), which was cloned into pBR 322 (kindly provided from C. Nozaki [Fujiyama et al., 1983]), was used for the quantification of HBV DNA copy numbers in serum. Sera were diluted serially 10-fold with distilled water and amplified by polymerase chain reaction (PCR) with nested primers according to the nucleotide sequence of the S gene [Iizuka et al., 1992]. The copy number in the sera was calculated from the comparison of the highest dilution (10^n) of the control HBV DNA and of HBV DNA from patients showing positive signals. Ten copies of HBV DNA were the theoretical limitation of the detection by PCR in our experimental conditions.

PCR and Nucleotide Sequencing

The target region of the present study consisted of core upstream and precore regions which overlap with the X gene, as described above, spanning 371 nucleotide lengths from nt. 1604 to nt. 1974. Template DNAs were extracted from serum (100 μl) by the sodium iodide method. The following synthetic oligonucleotide primers were prepared according to the reported sequences of the *adr* subtype [Fujiyama et al., 1983]: sense outer primer, named NRE (nt. 1481-1500, 5′-TCTACCGTCCCCTTCTTCAT-3′); sense inner primer, named Mβ(nt. 1604-1626, 5′-TCGCATGGAGACCACCGTGA-3′) and antisense primer, named BC1 (nt. 1953-1974, 5′-GGAAAGAAGTCAGAAGGCAA-3′). The first PCR was carried out by using a primer set of NRE and BC1 and the second nested PCR to amplify the target region of HBV DNA spanning 371 nucleotide length was carried out using Mβ and BC1 primers. PCR was undertaken under the following conditions: Briefly, 50 μl reaction mixtures containing 5 μl of sample DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 1 μM each of the two oligonucleotide primers, 200 μM dNTP, and 2 U of Taq polymerase were overlaid with mineral oil. The reaction mixtures were exposed to 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. The amplified product after the second PCR was purified after extraction from agarose gel and then subcloned into the pGEM-T vector (Promega Corp. Madison, WI). Six independent clones were isolated and sequenced by the dideoxy termination method using a thermal sequencing system (Perkin Elmer, Foster City, CA). Each PCR contained two negative controls replacing the template by water. All possible precautions were taken to avoid contamination in PCR in the following ways. All procedures of RNA extraction, thermal cycling of PCR and electrophoresis on agarose gel were performed in each separated room. All other manipulations were also carried out carefully by using disposable microtips and handgloves.

TABLE II. Transition of Mutant Clones*

Case	Mutant type	ASC	Exacerbation	Post-exacerbation
1	A	6†	0	0
	B	0	6	0
	C	0	0	6
2	A	4	1	1
	B	0	0	5
	C	1	5	0
3	A	1	0	
	B	0	0	not examined
	C	5	6	due to death

*Six independent clones from different phases of all cases were sequenced.
†No. of clones.

RESULTS

Sequential analyses of the target region in the HBV genomes obtained from three anti-HBe positive subjects revealed that there are several characteristic mutations specific to clinical phases. Table II summarizes the transition of mutant clones during the asymptomatic phase and exacerbation and post-exacerbation phases. In the asymptomatic carrier phase, the most dominant mutant, especially in cases 1 and 2, was an 8-bp deletion (nt. 1768-1775) (Fig. 1, type A mutation) resulting in a partial lack of basic core promoter (BCP) and in a frame shift attached to three novel amino acids before a novel stop codon in the X open reading frame (ORF). In case 3, the type A mutant was detected in only one out of 6 clones tested. Instead, the mutant possessing a precore stop codon mutation G to A (nt. 1896) with additional contiguous point mutations A to

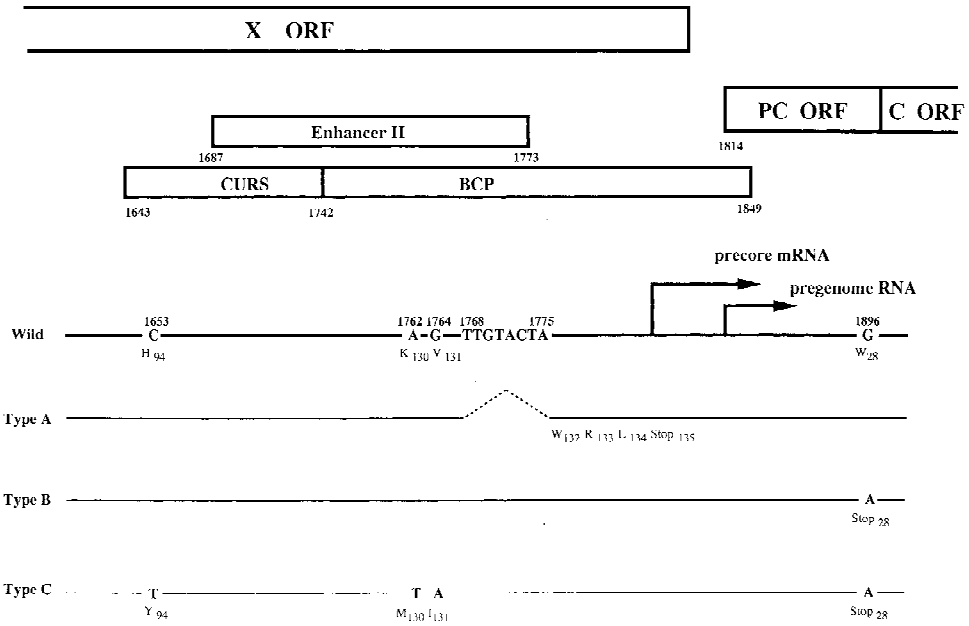

Fig. 1. Schematic diagram of the wild type *adr* subtype HBV DNA spanning from core upstream region to precore/core region as indicated by thick line. Upper boxes represent cis-acting elements [core promoter consisting of core upstream regulatory sequences (CURS) and BCP, enhancer II] and X-ORF and PC/C ORF. Two arrows indicate the initiation site of precore and pregenome RNAs. The numbers in bold type indicate nucleotide positions on the HBV genome. Lower thin lines indicate representative mutant HBV DNA (type A to C) and single letter codes and attached numbers below the lines are the deduced amino acid numbers from the *N*-terminus of the X protein substituted due to nucleotide mutation. The number attached to "stop" indicates the amino acid number from the *N*-terminus of precore protein.

T (nt. 1762) and G to A (nt. 1764) and a point mutation from C to T (nt. 1653) (Fig. 1, type C mutation) was dominant. However, the type A mutant disappeared after exacerbation as seen in cases 1 and 2. Case 3 appeared to be a unique case, because a blood sampling for nucleotide sequencing was performed 40 days after the start of predonisolone administration although the patient was clinically in the asymptomatic carrier phase. One possible explanation for this discrepancy might be that clinically silent progression of exacerbation and the resulting vigorous replication of type B mutant had already taken place in the patient at the time of the blood sampling and that the type A mutant might have been a dominant mutant as seen in cases 1 and 2 if the blood sampling had been performed before the start of the predonisolone treatment.

After exacerbation in case 1, the type A mutants were replaced exclusively with mutants possessing a precore stop codon mutation G to A at nt. 1896 (Fig. 1, type B mutation) in the exacerbation phase and these type B mutants were again replaced completely with type C in the post-exacerbation phase. However, in case 2, the transition of type B and type C mutants after exacerbation showed a contrary direction to that observed in case 1 and type C mutants were dominant in the acute exacerbation phase, but not in the post-exacerbation phase. In case 3, type C mutants were also dominant in the acute exacerbation phase as seen in case 2, although transition to the post-exacerbation phase could not be followed because the patient died.

Thus, although a definite transitional direction between type B and C mutants was not observed from acute exacerbation to post-exacerbation phases, the present study demonstrates that type A mutant might be a dominant mutant in the ASC phase and after exacerbation type B and C mutants appear and become dominant with decreasing populations of type A mutants.

Figure 2 shows the nucleotide sequences spanning 300 bps (nt. 1604-1903) lengths of the six independent clones obtained from the ASC phase (451-1 to 6), the exacerbation phase (452-1 to 6) and the post-exacerbation phase (458-1 to 6) of case 1. In the asymptomatic phase, all tested clones (451-1 to 6) showed only a type A mutation with complete homogeneity and did not possess other mutations. All clones from the exacerbation phase possessed a type B mutation as a common mutation. However, it consisted of diverse populations having different indefinite point mutations in contrast to those in the asymptomatic phase. In the clones, there were new mutations G to A and T to G at nts. 1748 and 1760, respectively. These mutations, however, did not result in the amino acid substitutions in the X ORF but had the possibility of modifying transcriptional activities because the mutations were located in BCP region. Finally, in the post-exacerbation phase, tested clones possessed a type C common mutation but again it consisted of heterologous clones having different point mutations. A new point mutation C to T at nt. 1653 found in a type C mutation was a sense mutation causing His_{94} to Thy_{94} in the X ORF. Another new point mutation A to T at nt. 1852 was observed in

Fig. 2. Nucleotide sequences spanning 300 bps (nt. 1604-1903) length of 6 independent clones obtained from ASC phase (A, clones 451-1 to 6), exacerbation phase (B, clones 452-1 to 6) and post-exacerbation phase (C, clones 458-1 to 6) in case 1 showing the core upstream, core promoter and precore/core regions. The top line is referred from the wild type HBV *adr* (GeneBank and EMBL, accession number X01587) sequence. A dot (.) indicate identical nucleotides with reference sequence and an asterisk (*) indicates common mutation in all 6 clones tested.

5 of 6 tested clones having a type C mutation but this mutation was a silent mutation in regard to the precore ORF.

DISCUSSION

The present study was designed to discover the sequential transition of the mutations occurring in the acute exacerbation and post-exacerbation phases from the asymptomatic carrier phase. We, therefore, analysed the nucleotide sequences of the HBV clones obtained from three different phases of three subjects who were positive for anti-HBe and showed acute exacerbation after immunosuppressive therapy. Most of the mutations found in the present study from these phases of hepatitis B have already been reported to exist in asymptomatic carriers by investigations dealing with only one point of various clinical phases [Fukuda et al., 1995; Hasegawa et al., 1994; Ogata et al., 1993; Okamoto et al., 1994]. To our knowledge, however, this is the first study to deal with the sequential analyses of mutations in the core upstream and precore regions of the hepatitis B virus genome through several different clinical phases.

Sequential transition from type A mutation in the ASC phase to type B or C mutations after exacerbation has been demonstrated in the present study. However, a possible definite transitional course of the mutation from acute exacerbation to post-exacerbation was not observed between type B and C mutations having a common stop codon mutation G to A (nt. 1896). This might be ascribed to the subtle difference of blood sampling time, different background diseases of the subjects, different immunosuppressive drugs and the period of administration, or other factors. Therefore, further studies with increasing numbers of patients are necessary to make clear the possible relationship between the mutation and clinical course, although such acute exacerbated case after immunosuppressive therapy as those dealt with in the present study are very rare.

The core upstream region contains the pregenomic RNA start site [Yaginuma et al., 1987], core promoter/enhancer II [Yee, 1989; Yuh et al., 1992] and some nuclear factor binding sites [Zhang et al., 1994], all of which appear to be important in the viral life cycle. However, our previous study revealed that the mutations at nt. 1762 and 1764 had no apparent effects on promoter and enhancer activities [Nishizono et al., 1995].

Eight-bp deletion mutation (type A mutation) might have the potential of reducing promoter or enhancer activity because the mutation is located in the center of BCP/enhancer II. Recent research has shown that AT-rich sequences spanning a 15 nt. stretch which is located in the latter part of BCP (nt. 1788-1802:CATAAATTGGTCTGT) is sufficient to direct precise initiation of both precore mRNA and pregenome RNA syntheses. [Chen et al., 1995]. These observations might suggest that 8-bp deletion at nt. 1768-1775 or contiguous point mutations A to T and G to A at nts. 1762 and 1764 are not involved in the precise initiation of precore or pregenome RNA syntheses.

On the other hand, the core upstream region not only contains *cis*-acting transcriptional regulatory elements but also encodes the transacting-X protein. Since the X protein has been suggested to activate transcription by catalyzing the phosphorylation of cellular factors [Wu et al., 1990], the 8-bp deletion resulting in a *C*-terminal truncation might lose its transacting function and lead to the reduction of the synthesis of HBV transcripts. In our preliminary observations, the level of mRNA synthesis of the HBV genome having 8-bp deletion apparently decreased when compared with that of the wild type HBV genome.

As described above, the ability of these mutants to replicate appears to be suppressed, in an opposite direction to reactivation. Thus viral reactivation mechanisms would not be ascribed simply to these mutations but rather to other aspects such as the effect of the decrease or disappearance of mutant clones possessing 8-bp deletion in early exacerbation phase, the involvement of new mutations at nt. 1653 (C to T), and the stimulation by immunosuppressive drugs of the glucocorticoid responsive element which has been shown to induce viral propagation [Tur-Kaspa et al., 1988].

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